Nematode-Mycobiota Interactions in Pine Wilt Disease

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PineEnemy

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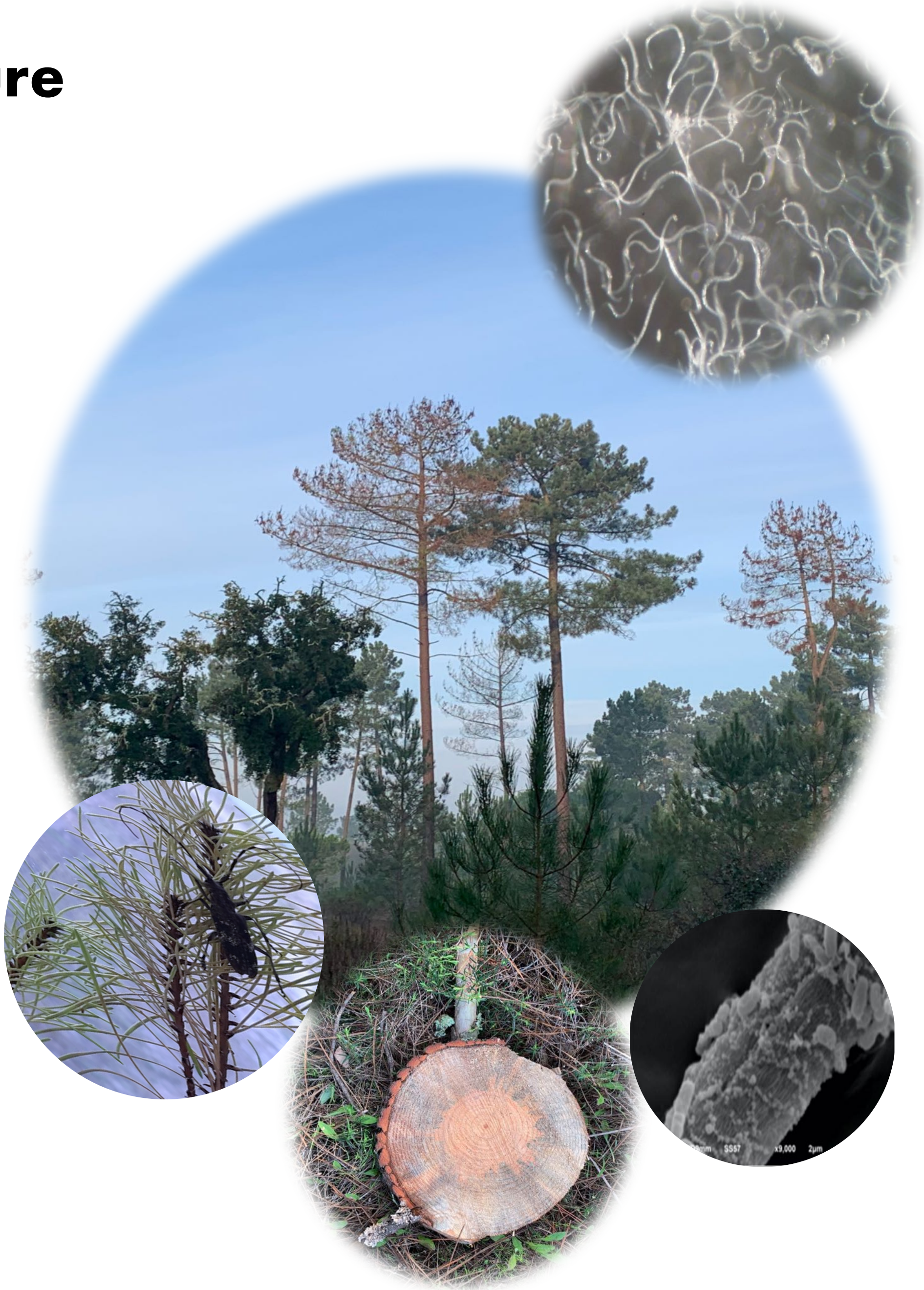
Forests communities in nature

- **Forest trees** harbour extremely complex communities that play important roles on ecosystem **multifunctionality and equilibrium**.

- Under the **presence of non-native pathogens**, these ecosystems suffer shifts that can eventually lead to **considerable spatial and temporal communities variation**.

- In the context of PWD, the **presence of *Bursaphelenchus xylophilus*** causes **imbalances in the communities associated with the host tree**.



Nematode – Fungi Interaction

- Description of fungal communities has been made in different pine species, insect-vectors and PWN cultures across **China, Japan, Korea, USA and Portugal**;
- Most of the described taxa are **common saprophytes** and probably **not specific to the disease** or **associated to PWN**;
- Recent HTS studies showed that **fungal community richness and diversity decreases** with the increase of disease disturbance – **presence of PWN**.

Table 1. Culture-dependent mycoflora isolated from the PWN, insect-vectors, and host pines. PWN, *Bursaphelenchus xylophilus*; Ma, *Monochamus alternatus*; Ms, *M. scutellatus*; Mg, *M. galloprovincialis* (A—Adult, L—Larvae); Pd, *Pinus densiflora*; Pm, *P. massoniana*; Pt, *P. thunbergii*; Pb, *P. banksiana*; Pr, *P. resinosa*; Pp, *P. pinaster* (W—wood, Pc, pupal chamber); JP, Japan; PT, Portugal; KR, Korea; CH, China; USA, United States of America.

Fungal Taxonomy					Insect-Vector						Host Pine								Country	References
					Ma		Ms		Mc	Mg	Pd		Pm	Pt		Pb	Pr	Pp		
Phylum	Class	Family	Genus	PWN	A	L	A	L	A	A	W	Pc	Pc	W	Pc	Pc	Pc	W		
Ascomycota	Blastomycetes	Crytococcaceae	*Candida*								•								JP	[52]
	Dothideomycetes	Botryosphaeriaceae	*Diplodia*								•			•					JP	[39,52]
			Botryosphaeria							•									PT	[46]
			Macrophoma								•								JP	[53]
			Sphaeropsis											•					JP	[54]
		Cladosporiaceae	*Cladosporium*		•					•	•			•				•	JP, PT	[39,43,45,46,52]
		Didymellaceae	*Epicoccum*							•								•	PT	[45,46]
		Dothideaceae	*Rhizosphaera*								•									[39]
		Leptosphaeriaceae	*Leptosphaeria*		•														KR	[44]
		Massarinaceae	*Helminthosporium*				•	•											USA	[42]
		Pleosporaceae	*Alternaria*	•	•		•	•		•	•							•	JP, PT	[39,45,46,52]
			Bipolaris							•									PT	[45]
			Curvalaria	•															CH	[55]
			Stemphylium					•											PT	[45]
		Saccotheciaceae	*Aureobasidium*				•	•		•				•					JP, PT	[45,46,54]
	Eurotiomycetes	Aspergillaceae	*Aspergillus*	•	•		•	•		•	•			•				•	JP, CH, PT	[42,44–46,52,54,55]
		Trichocomaceae	*Penicillium*	•	•	•	•	•		•	•			•	•	•	•	•	JP, CH, PT	[39,44–46,52–54]
			Paecilomyces							•								•	PT	[45,46]
		Herpotrichiellaceae	*Phialophora*											•		•	•		JP	[42,52]
			Rhinocladiella													•	•		USA	[42]
	Leotiomycetes	Sclerotinuaceae	*Botrytis*							•					•			•	CH, PT	[44–46]
	Orbiliomycetes	Orbiliaceae	*Arthrobotrys*	•							•					•	•		JP, CH, USA	[42,52,53]
			Dactylaria								•								JP	[52]
	Sordariomycetes	Amphisphaeriaceae	*Pestalotia*		•														JP	[39]
		Aspiosporaceae	*Arthrinium*							•								•	PT	[45,46]
		Bionectriaceae	*Bionectria*												•				KR	[44]
			Clonostachys							•								•	PT	[45,46]
		Boliniaceae	*Camarops*												•				KR	[44]
		Ceratocystidaceae	*Ceratocystis*	•	•		•	•	•		•	•		•		•	•		JP, CH, USA	[39,42,44,53,55]
		Chaetomiaceae	*Chaetomium*	•															CH	[55]
		Cordycipitaceae	*Beauveria*				•	•		•								•	USA, PT	[42,45,46]
		Glomerellaceae	*Colletotrichum*	•	•		•	•											JP, CH	[39,55]
		Hypocreaceae	*Hypocrea*		•										•				KR	[44]
			Cephalosporium								•								JP	[52]
			Gliocladium											•		•	•		JP, USA	[42,54]
			Trichoderma	•			•	•		•	•			•	•	•	•	•	JP, CH, USA, PT	[39,42,44–46,52–55]
		Microascaceae	*Graphium*								•								JP	[52]
		Nectriaceae	*Gibberella*		•														KR	[44]
			Fusarium	•						•	•			•	•			•	JP, CH, KR, PT	[39,44–46,53–55]
			Mariannaea								•								JP	[52]
			Nectria												•				KR	[44]
		Ophiostomataceae	*Ceratocystiopsis*				•	•	•							•	•	•	USA, PT	[42,46]
			Graphilbum										•	•				•	KR, PT	[46,47,50]
			Leptographium								•								JP	[39,53]
			Ophiostoma	•	•	•				•			•	•	•			•	CH, KR, PT	[44–47,56]
			Sporothrix										•	•				•	CH, PT	[46,47]
		Plectosphaerel-laceae	*Plectosphaerella*		•														KR	[44]
			Verticillum	•										•					JP	[53]
		Sordariaceae	*Sordaria*	•															CH	[55]
		Sporocadaceae	*Monochaetia*	•															CH	[55]
			Pestalotiopsis	•							•			•				•	JP, CH, PT	[43,46,52–55]
		Trichosphaeriaceae	*Nigrospora*	•										•					JP, CH	[43,55]
		Xenospadicoidaceae	*Spadicoides*												•	•			USA	[42]
		Valsaceae	*Phomopsis*	•							•			•					JP	[52,53,55]
		Incertae sedis	*Trichothecium*							•								•	PT	[45,46]
Basidiomycota	Agariomycetes	Irpicaceae	*Irpex*		•														KR	[44]
		Ceratobasidiaceae	*Rhizoctonia*											•					JP	[54]
Mucoromy-cota	Mortierellomy-cetes	Mortierellaceae	*Mortierella*											•					JP	[54]
	Mucoromycetes	Mucoraceae	*Mucor*	•										•	•				KR	[44]
		Rhizopodaceae	*Rhizopus*	•															CH	[55]

Nematode – Fungi Interaction: the case of Ophiostomatales

Fungal Taxonomy					Insect-Vector						Host Pine								Country
					Ma		Ms		Mc	Mg	Pd		Pm	Pt		Pb	Pr	Pp	
Phylum	Class	Family	Genus	PWN	A	L	A	L	A	A	W	Pc	Pc	W	Pc	Pc	Pc	W	
Ascomycota	Sordariomycetes	Ophiostomataceae	*Ceratocystiopsis*				•	•	•							•	•	•	USA, PT
			Graphilbum										•	•				•	KR, PT
			Leptographium								•								JP
			Ophiostoma	•	•	•				•			•	•	•			•	CH, KR, PT
			Sporothrix										•	•				•	CH, PT

Vicente et al., 2021, JoF 7:780

Blue-stain fungi
(Image taken on January 3rd, 2021)

- Beetle-associated fungi from unrelated orders Ophiostomatales and Microscales (*Ceratocystis*) are mostly necrotrophic pathogens able to colonize the phloem and xylem of pine trees;

- Reproduction structures of some blue-stain fungi were detected in *Monochamus* sp. tunnels, as well as pine trees or nematode – association with the disease;

- Several studies showed the relation between the presence of Ophiostomatales and the dense multiplication of PWN, as well as the number of PWN transferred to *Monochamus* sp. (Maehara and Futai, 1996, 1997, 2000; Maehara et al., 2006; Maehara, 2008);

- Other studies suggested that PWN uses high monoterpene concentrations and native blue-stain fungi to improve its propagation (Niu et al. 2012)

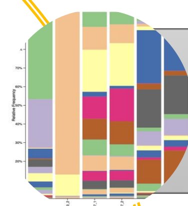
Study Aims

PineEnemy

Exploring the NEmatode-MYcobiota interactions in Pine Wilt Disease

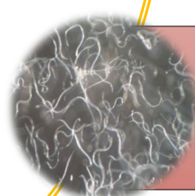
https://twitter.com/EnemyPine
https://projects.iniav.pt/pineenemy/

Understand if **nematode-associated mycoflora plays a key-role in the development of the disease**, in interaction with nematode and insect-vector, and into **which extend it can be used to disrupt the disease cycle**.



Identification of PWD-associated fungal communities through metagenomic analysis

Characterization of candidate fungi (Ophiostomatales) for PWN feeding



Effect of PWN specific associated fungi on the nematode life-cycle

Methodology

Experimental Design

Three sampling locations in Portugal PWD foci

All samples were processed for PWN presence/quantification

Metagenomic Approach

Optimization of total DNA extraction from wood (w/wo PWN)

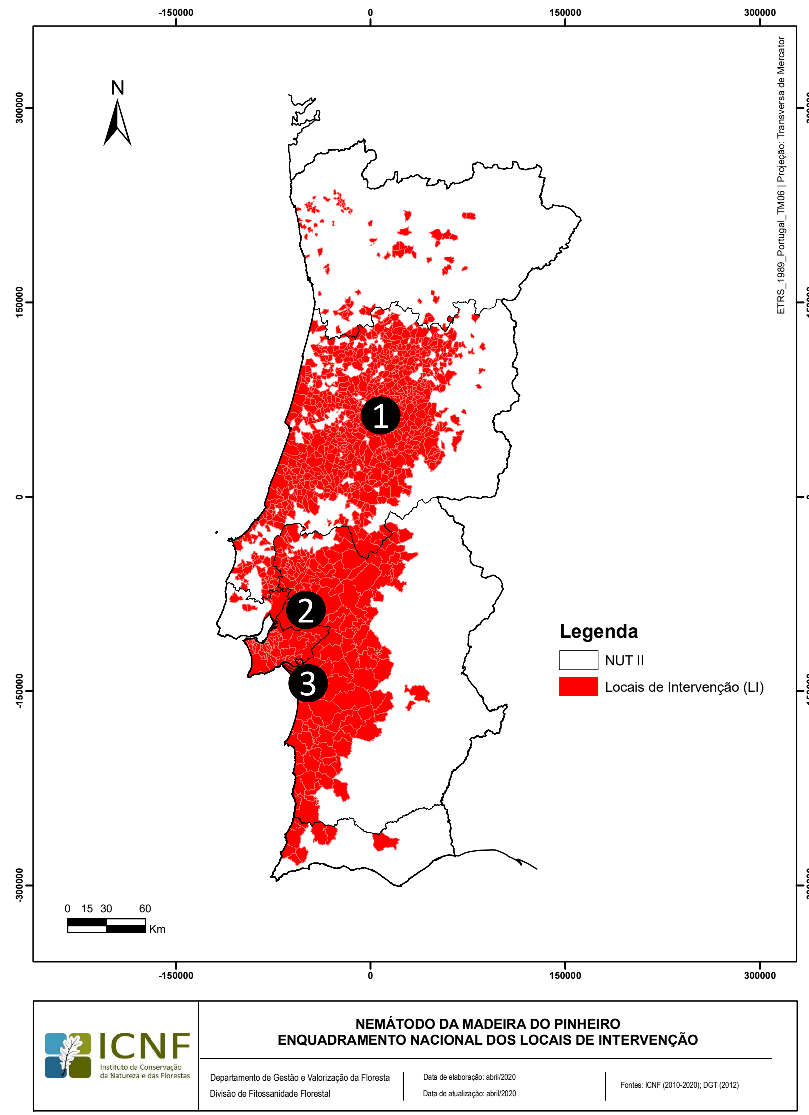
Sequencing of ITS2 amplicon and bioinformatic analysis

Culturomics Approach

Isolation of fungal communities associated with *P. pinaster* infected and non-infected with the PWN

Feeding trials with Ophiostomatales

Evaluation of PWN on different Ophiostomatales species

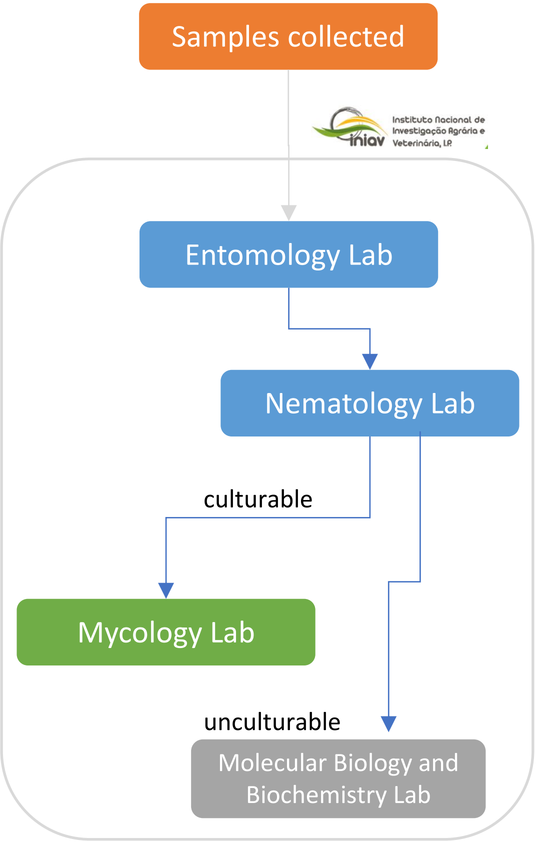


ICNF, Updated data from 2020

Sampling processing

Collection sites						*Pinus pinaster* samples			
Location	GPS	Tmax (ºC)[1]	Tmin (ºC)[1]	Precipitation (mm)[1]	Altitude (m)	*PWD symptoms*	Tree ID	PWD symptoms[2]	PWN class[3]
Seia (S)	40°15'57.0"N 7°42'47.6"W	18	0.5	50-100	701	No	S1	0	0
							S2	0	0
							S13	0	0
							S19	0	0
							S32	0	0
						Yes	S9	3	IV
							S36	3	IV
							S38	3	IV
							S39	3	III
							S40	3	III
Companhia das Lezírias (CL)	38°49'17.6"N 8°52'20.5"W	10	1.5	50-100	38	No	CL7	0	0
							CL8	0	0
							CL9	0	0
							CL10	0	0
							CL11	0	0
						Yes	CL2	4	III
							CL3	3	III
							CL4	3	IV
							CL5	3	III
							CL6	4	IV
Tróia (T)	38º28'07''N 8º52'18''W	20	0.5	1-5	359	No	T8	0	0
							T9	0	0
							T10	0	0
							T11	0	0
							T12	0	0
						Yes	T1	4	IV
							T2	4	III
							T3	4	II
							T4	4	III
							T7	4	IV

(1) Average values at collection time; **(2) Symptoms Class**: 0, no symptoms; 1, one yellow branch; 2, yellow canopy; 3, yellowish to reddish canopy; 4, brown canopy - dead tree. **(3)** Nematode Class (per 100 grams of wood): 0, no nematodes; I, < 50 nematodes; II, 51 to 200 nematodes; III, 201 to 1000 nematodes; IV, 1001 to 5000 nematodes; V, > 5001 nematodes.



- Surveys were conducted before maturation feeding phase of the insect-vector *Monochamus galloprovincialis*.

PWD-associated fungal community: metagenomic approach – preliminary data

Location	Sample ID	Presence of PWN	DNA extraction method
Seia	S40_2	YES	QIAGEN DNeasy
Seia	S2_2	NO	QIAGEN DNeasy
Troia	T7_1	YES	CTAB modified
Troia	T7_2		QIAGEN DNeasy
Troia	T8_1	NO	CTAB modified
Troia	T8_2		QIAGEN DNeasy

- Data set: 158,325 ITS2 reads from 6 samples ranging between 8545 (sample T8_1) to 75629 reads (S2_2);
- Few differences between the two extractions methods;
- Close-reference clustering (at 97% sequence similarity cutoff) produced a range of **40-65 operational taxonomic units (OTUs) for *P. pinaster* trees infected with PWN** and **103-116 OTUs for *P. pinaster* without PWN**;
- Fungal communities from Seia showed higher diversity than fungal communities of Troia.
- Core metrics also suggest that the presence of PWN may alter the fungal community structure.

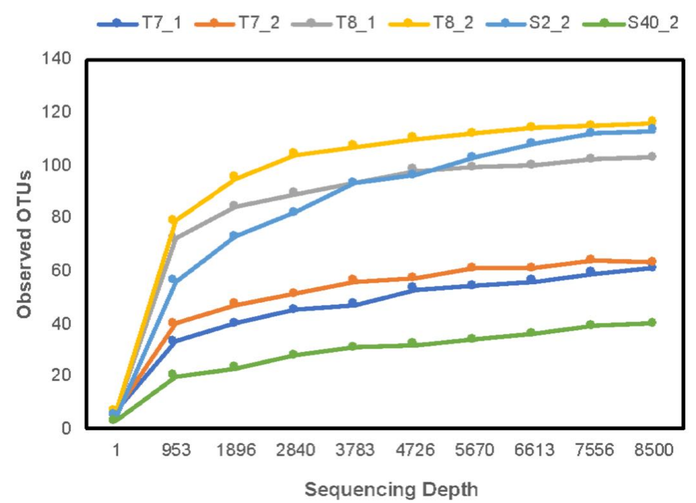
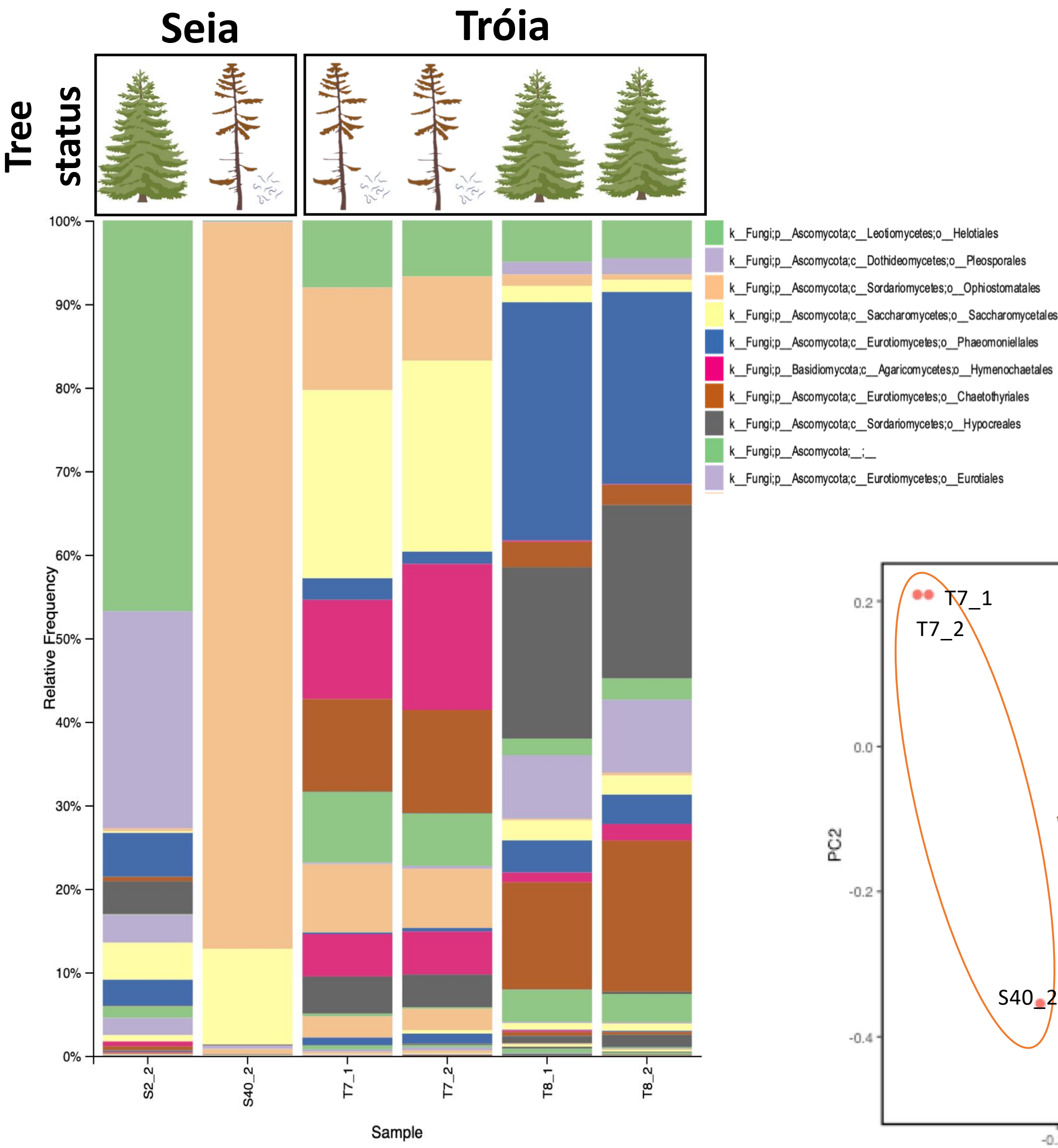


FIGURE 1|Accumulation curves of fungal mycobiomes per sample. Sequencing depth was normalized at 8500 features per sample.

TABLE 1|Core metrics (diversity indices) calculated for each sample from collection sites Troia (T) and Seia (S).

Core Metrics	Presence of PWN			Absence of PWN		
	T7_1	T7_2	S40_2	T8_1	T8_2	S2_2
Chao1	70	68	53	106	118	135
Observed OTU	62	65	40	103	116	112
Simpson index	0.93	0.92	0.60	0.90	0.91	0.74
Shannon Entropy	4.06	4.06	1.84	4.42	4.51	3.12
Pielous J	0.68	0.68	0.34	0.66	0.66	0.46
Good Coverage	0.99	0.99	0.99	0.99	0.99	0.99



Not only the communities differ between study sites, which are biogeographically distinct, but also due to the presence of the nematode.

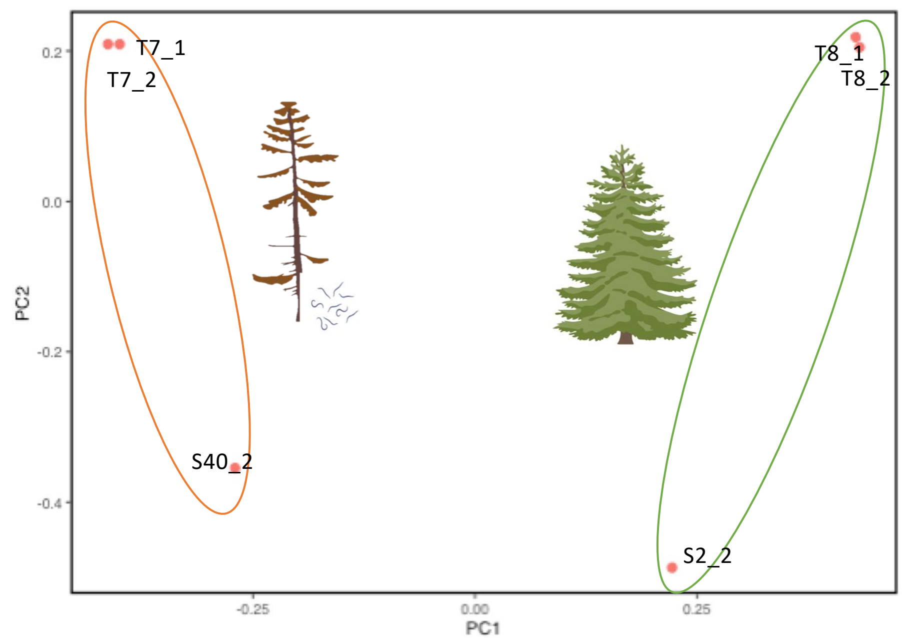


FIGURE 2|Relative frequency of fungal communities (level order) of *Pinus pinaster* trees infected with PWN (showing visible PWD symptoms) and without PWN.

FIGURE 3|Principal coordinate analysis **(**PCoA) based on Bray-Curtis distance matrix of 6 samples (2 *Pinus pinaster* from Seia and 4 *P. pinaster* from Troia.

PWD-associated fungal community: culturomics

- Total number of fungal isolations: 242 isolates were obtained from *P. pinaster* trees (45 from **2**; 94 from **3**; and 103 from **1**) and 80 isolates from the emerged *M. galloprovinciallis* (48 from **1**; 30 from **3**; and 3 from **2**);

- Fungal communities shifts between *P. pinaster* infected and non-infected with PWN;

- Total number of sequences for phylogenetic inference: 366 (126 **ITS2**; 84 **TUB**; 89 **TEF**; 67 **CAL**) distributed from 51 (**2**), 203 (**1**) and 112 (**3**).

TABLE 2| Core metrics (diversity indices) calculated for each sample from collection sites Troia (T) and Seia (S).

Diversity Indices	By Location x PWN detection					
	Seia (1)		Lezirias (2)		Troia (3)	
	No PWN	PWN	No PWN	PWN	No PWN	PWN
Taxa_S	6	4	4	6	6	5
Individuals	43	60	25	20	44	50
Dominance_D	0.2872	0.535	0.36	0.255	0.469	0.3096
Simpson_1-D	0.7128	0.465	0.64	0.745	0.531	0.6904
Shannon_H	1.434	0.8711	1.139	1.51	1.106	1.276
Evenness_e^H/S	0.6989	0.5974	0.7808	0.7544	0.5038	0.7162
Chao-1	6	4	4	9	6.5	5

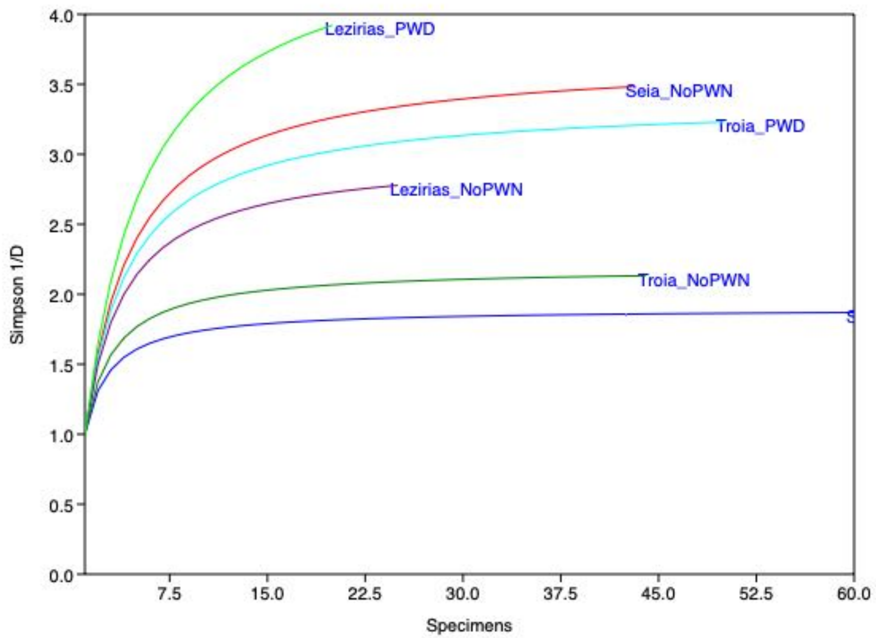


FIGURE 4| Accumulation curves of fungal communities per sample.

Vicente et al., 2021 (*in preparation*)

PWD-associated fungal community: culturomics

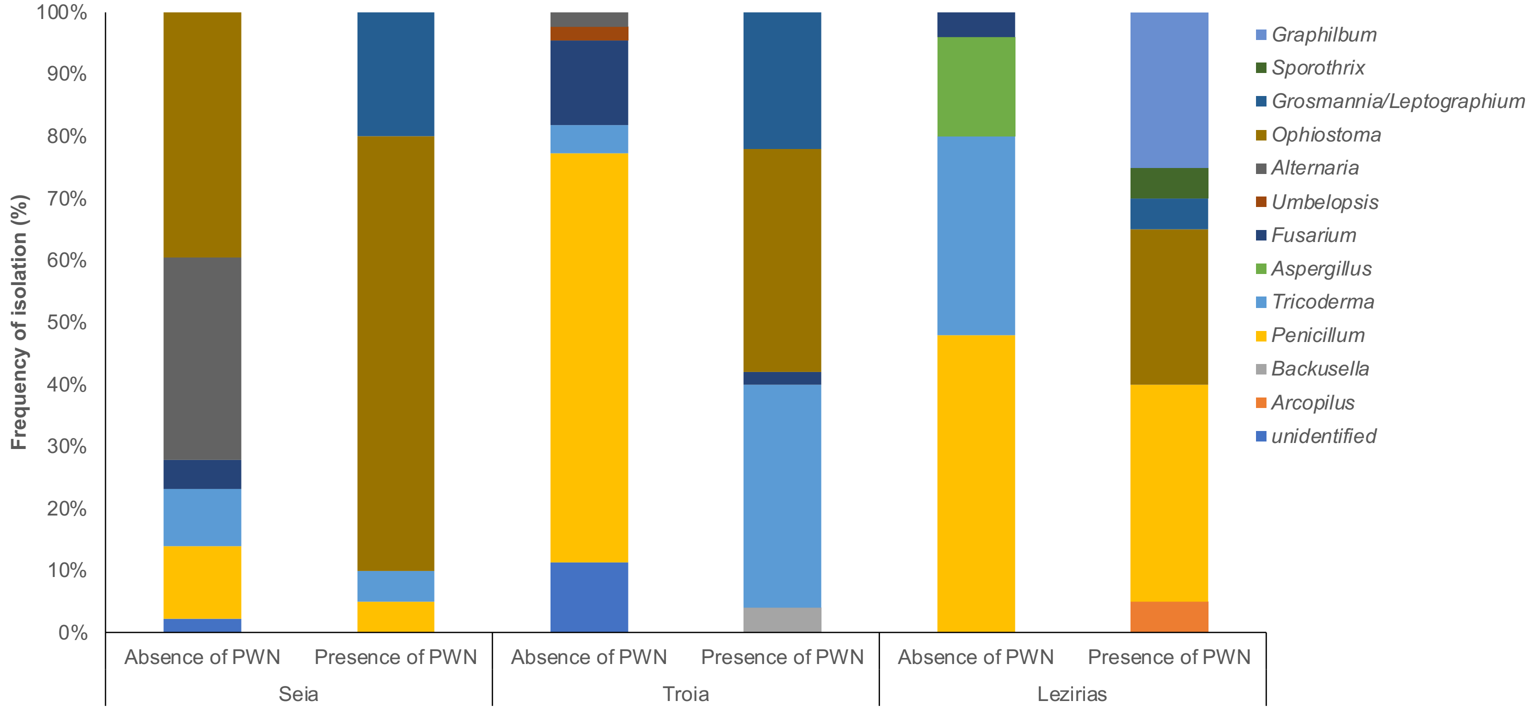
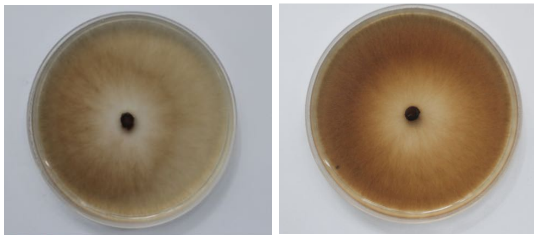


FIGURE 5 | Frequency of isolation of fungal genera in each collection site.

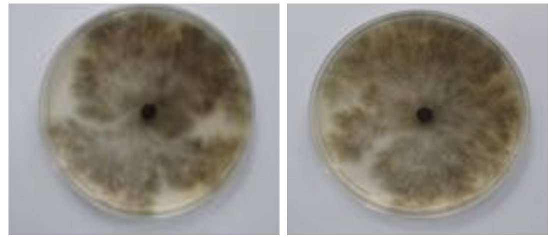
- In overall, dominance of Ophiostomatales (*Ophiostoma ips* > 50% FI) in *P. pinaster* infected with PWN;
- In exception of 1 tree in Seia (1), Ophiostomatales were only present in *P. pinaster* infected with PWN;
- *P. pinaster* without PWN (healthy trees) presented more diverse fungal community than *P. pinaster* with PWN.

Not all Ophiostomatales are suitable for PWN multiplication

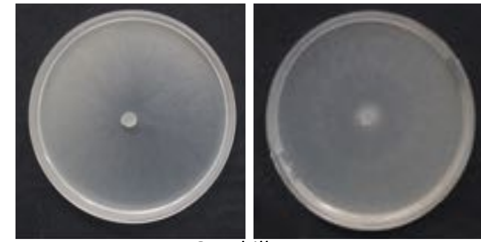
Data and images obtained by Miguel Soares (Master Student)



Ophiostoma ips



Leptographium terebrantis



Graphilbum sp.

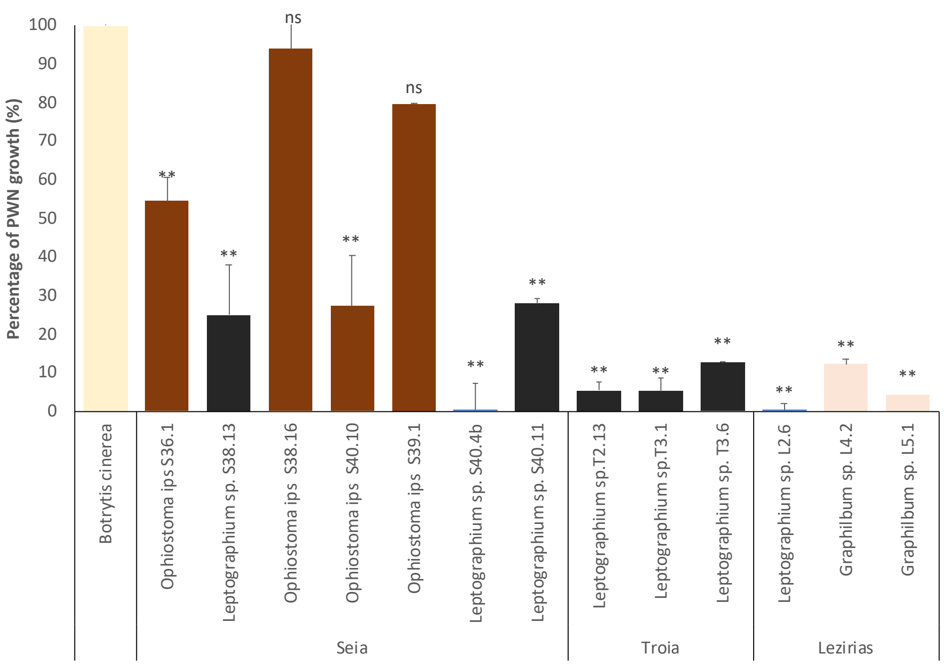
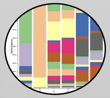


FIGURE 6|Effect of Ophiostomatales in PWN growth in comparison with *Botrytis cinerea*. Asterisks on top of columns indicate significant diferences at 99% (**) of confidence.

- Intraspecific variability between fungi isolates;
- PWN growth in *Ophiostoma ips* S38.16 and S39.1 was closest to growth in *B. cinerea* (p>0.001);
- All *Leptographium terebrantis* and *Graphilbum* sp. isolates were not suitable for PWN (in comparison with *B. cinerea*, p<0.001);
- Almost no PWN growth in *Leptographium* sp. S40.4b and L2.6;
- **Question raised:** was PWN concentration high in *P. pinaster* trees infected by *Leptographium* or *Graphilbum* (mostly from Tróia and Lezírias collection sites)?

Conclusions

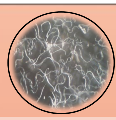


Metagenomic Approach

Presence of PWN alters fungi community composition and structure.

Culturomics Approach

Fungal communities shifts between *P. pinaster* infected and non-infected with PWN



Feeding trials with Ophiostomatales

Not all Ophiostomatales are suitable for PWN multiplication (in particularly *Leptographium terebrantis* and *Graphilbum* sp.)

Explore the biocontrol potential of these Ophiostomatales isolates, as well as, other already described nematophagous fungi such as *Esteya vermicola* (PhD student David Pires).

Acknowledgments

NemaINIAV, INIAV I.P. : Marina Cardoso and Miguel Soares (not in the picture)

Molecular Biology & Biochemistry Lab, INIAV I.P. : Filomena Nóbrega, Rita Varela

Mycology Lab , INIAV I.P. : Helena Bragança, Florinda Medeiros

Entomology Lab, INIAV I.P. : Edmundo Sousa, Luís Bonifácio, Pedro Naves, Adérito Sousa


NemaLab, MED-UE: Manuel Mota e Margarida Espada

Funding:

PineEnemy

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

GOVERNO DE PORTUGAL

Partners and Consultants:

iniav Instituto Nacional de Investigação Agrária e Veterinária, I.P.

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